

Remarks

Claims 46-66 were pending in the subject application. By this Amendment, claims 46-66 have been canceled and new claims 67-94 have been added. The undersigned avers that no new matter is introduced by this amendment. Entry and consideration of the amendments presented herein is respectfully requested. It should be understood that the amendments presented herein have been made solely to expedite prosecution of the subject application to completion and should not be construed as an indication of Applicants' agreement with or acquiescence in the Examiner's position. Accordingly, claims 67-94 are currently before the Examiner for consideration. Favorable consideration of the pending claims is respectfully requested.

As an initial matter, the Examiner has objected to the term "ligand" as used in the specification. Applicants submit that the term "ligand" is generally understood in the art to be a molecule that binds to another molecule. In the context of this application, examples of ligands to the claimed polypeptides include antibodies (see specification at page 15, lines 2-8). Accordingly, reconsideration of the objection to this term in the specification is respectfully requested.

Claim 46 has been objected to for encompassing non-elected subject matter. In view of the amendments presented in this response, it is respectfully submitted that this issue is now moot and reconsideration and withdrawal of the objection is respectfully requested.

Claims 46, 65, and 66 have been rejected under 35 USC §101 because the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility. The Office Action also rejects claims 46, 65, and 66 under 35 USC §112, first paragraph, as non-enabled by the subject specification on the basis that one skilled in the art would not know how to use the claimed polypeptides as they are now supported by a specific and substantial asserted utility or a well established utility.

The Office Action argues that the asserted therapeutic or preventative uses for the claimed polypeptides based on sequence homology to a known molecule are not considered substantial as generally the art acknowledges that the function of a protein cannot be predicted based solely on structural similarity to a known protein. In support of its position, the Office Action argues that, in the transforming growth factor (TGF) family, Vukicevic *et al.* (1996, *PNAS USA* 93:9021-9026)

discloses that OP-1, a member of the TGF- β family of proteins, has the ability to induce metanephrogenesis, whereas closely related TGF- β family members BMP-2 and TGF- 1 had no effect on metanephrogenesis under identical conditions (page 9023, paragraph bridging columns 1-2). In another example, IL- 18 receptor (IL-18R) was thought to be another IL-1 receptor (IL-1R) based on the sequence homology, and therefore, designated IL- 1 receptor-related protein (IL- 1Rrp) when it was first discovered and its ligand was unknown (Parnet *et al.*, *J. Biol. Chem.*, 1996, 271(8): 3967-70). The IL-1Rrp is now known as IL-18 receptor, has distinct ligand, and possesses distinct functional properties from that of IL-1R even though it is a member of the IL-1R family. The Office Action further argues that structural similarity does not necessarily indicate functional similarity, and in the instant case, the established utility for IL-8 cannot be automatically applied to said IL-8 like chemokine in the absence of any supporting evidence. Applicants respectfully traverse.

Applicants also note that Pisabarro *et al.* (*J. Immunol.*, 2006, 176:2069-2073, a copy of which is attached hereto) reported the identification of a chemokine using similar protocols to those disclosed herein. As noted in that reference, a fold recognition algorithm was utilized to identify and characterize a novel chemokine-like protein (DMC) (see Abstract and Materials and Methods, pages 2069-2070). This system predicted DMC to have an IL-8-like chemokine fold and to be structurally and functionally related to CXCL8 and CXCL14. Pisabarro *et al.* further report that, consistent with their predictions, DMC induces migration of monocytes and immature dendritic cells (see Abstract and pages 2071-2072).

Pisabarro *et al.* is relevant to the instant claims as Applicants have utilized a system similar to that reported in the reference. As set forth in the as-filed specification, a sophisticated proprietary system "GENOME THREADER" was used to assist the functional annotation of the protein (see Example 2). The system used (GENOME THREADER) to identify the claimed polypeptides as members of the IL-8 family of polypeptides relies on sequence homologies, structural homologies and other relationships (such as taxonomical information) and utilizes this information in a sophisticated manner in order to assign a functional annotation.

As indicated in the as-filed specification, GENOME THREADER was used to identify/annotate the sequences claimed herein (see paragraph bridging pages 14-15). As indicated in Example 2 and Figure 3, the system predicted, with 65% confidence, that INSP094 has a protein

fold that is similar to *H. sapiens* macrophage inflammatory protein 1 beta (MIP-1 β) and Figure 4 shows an alignment to these two proteins in which the conserved cysteine residues are highlighted. As the Patent Office will note, the aligned polypeptides exhibit conserved cysteine motifs that are consistent with CC chemokines. Thus, the prediction of IL-8-like activity for the claimed polypeptide is based upon more than structural similarity to a known protein and is based upon structural information that does not rely, solely, on sequence comparisons. Further, it is respectfully submitted that one skilled in the art would have recognized, based upon the examples provided in the as-filed application, that the claimed polypeptides would have been expected to function in a fashion similar to MIP-1 β .

As the Patent Office is aware, the predecessor court of the Federal Circuit has also held that the identification of a pharmacological activity of a compound that is relevant to an asserted pharmacological use provides an “immediate benefit to the public” and thus satisfies the utility requirement. In *Nelson v. Bowler*, 626 F.2d 853, 206 U.S.P.Q. 881 (1980), the Court of Customs and Patent Appeals, stated that knowledge of the pharmacological activity of any compound is obviously beneficial to the public. The Court of Appeals for the Federal Circuit has also found utility for therapeutic inventions despite the fact that the invention may be at a very early stage in the development of a pharmaceutical product or therapeutic regimen based on a claimed pharmacological or bioactive compound or composition. In *Cross v. Iizuka*, 753 F.2d 1040, 1051, 224 U.S.P.Q. 739, 747-48 (Fed. Cir. 1985), stated:

We perceive no insurmountable difficulty, under appropriate circumstances, in finding that the first link in the screening chain, *in vitro* testing, may establish a practical utility for the compound in question. Successful *in vitro* testing will marshal resources and direct the expenditure of effort to further *in vivo* testing of the most potent compounds, thereby providing an immediate benefit to the public, analogous to the benefit provided by the showing of an *in vivo* utility.

Accordingly, it is respectfully submitted that the asserted activity of the claimed polypeptide as an IL-8-like chemokine is a specific and substantial utility and that one skilled in the art would have found the asserted utility credible and known how one was to use the claimed polypeptides, particularly in view of the disclosure in the as-filed specification that the claimed polypeptide is expected to have a fold similar to that of MIP-1 β (see Example 2). Further, Applicants note that

MIP-1 β is art-recognized to be a monokine with inflammatory and chemokinetic properties. It is also one of the major HIV-suppressive factors produced by CD8⁺ T-cells and recombinant MIP-1-beta induces a dose-dependent inhibition of different strains of HIV-1, HIV-2, and simian immunodeficiency virus (SIV). Applicants respectfully submit that one skilled in the art would have recognized that the claimed polypeptides, more likely than not, would function in a fashion similar to MIP-1 β and the use of such polypeptides would have also been well-known to those skilled in the art. Accordingly, reconsideration and withdrawal of the rejection of record is respectfully requested.

Claims 46, 65, and 66 have been rejected under 35 USC §112, first paragraph, as non-enabled by the subject specification. The Office Action argues that the as-filed specification fails to enable functional equivalents of the claimed polypeptides. Further, the Office Action argues that the claims encompass an unreasonable number of distinct molecules, and inoperative polypeptides, that the specification provides no guidance or working examples as to how the skilled artisan could make the encompassed functional equivalents, or use inactive variants/fragments of SEQ ID NO: 10 or 12, as no functional limitation is associated with the variants in the claims. The Office Action continues that due to the large quantity of experimentation necessary to generate the infinite number of functional equivalents recited in the claims and possibly screen the same for activity (if the specification had disclosed a functional property for the INSP094 polypeptides), and to determine how to use the inoperative polypeptides, the lack of direction/guidance presented in the specification regarding same, the absence of working examples directed to same, the complex and unpredictable nature of the invention, and the breadth of the claims which embrace a broad class of structurally unrelated molecules, and structurally diverse variants without functional limitation, undue experimentation would be required of the skilled artisan to make and/or use the claimed invention in its full scope. Applicants respectfully traverse.

Enablement is a legal determination of whether a patent enables one skilled in the art to make and use the claimed invention (*Raytheon Co. v. Roper Corp.*, 724 F.2d 951, 960, 220 U.S.P.Q. 592, 599 (Fed. Cir. 1983)) and is not precluded even if some experimentation is necessary. *Atlas Powder Co. v. E.I. Du Pont De Nemours & Co.*, 750 F.2d 1569, 1576, 224 U.S.P.Q. 409, 413 (Fed. Cir. 1984); *W.L. Gore and Associates v. Garlock, Inc.*, 721 F.2d 1540, 1556, 220 U.S.P.Q. 303, 315 (Fed. Cir. 1983). Applicants also submit that nothing more than objective enablement is required, and

therefore, it is irrelevant whether this teaching is provided through broad terminology or illustrative examples. Additionally, the Patent and Trademark Office Board of Patent Appeals and Interferences has stated: “The test [for enablement] is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the invention claimed”. *Ex parte Jackson*, 217 U.S.P.Q. 804, 807 (1982); *see also Ex parte Erlich* 3 U.S.P.Q.2d 1011 (B.P.A.I. 1982) (observing that although a method might be “tedious and laborious,” such experimentation is nevertheless “routine” defining “routine” experiments as those which use known methods in combination with the variables taught in the patent to achieve the expected, specific, patented result).

With respect to the rejection of record, it is respectfully submitted that the as-filed specification provides adequate teachings as to how one skilled in the art is to make and/or test polypeptides within the scope of the instant claims. For example, methods of making functionally equivalent polypeptides are disclosed at pages 12-14 of the as-filed specification. Further, methods of screening the claimed polypeptides for biological activity are also disclosed in the as-filed specification at pages 54-56. Thus, while making and screening polypeptides within the scope of the claims might be tedious and labor intensive, such experimentation would be routine for those skilled in this area of technology, given the state of the art with respect to chemokines and chemokine research. Accordingly, reconsideration and withdrawal of the rejection of record is respectfully requested.

Claim 46 has been rejected under 35 USC §112, first paragraph, as lacking sufficient written description. The Office Action argues that to provide adequate written description and evidence of possession of a claimed genus, the specification must provide sufficient distinguishing identifying characteristics of the genus. The factors to be considered include disclosure of complete or partial structure, physical and/or chemical properties, functional characteristics, structure/function correlation, methods of making the claimed product, or any combination thereof. In this case, with respect to functional equivalents, none of the factors is present in the claim as the functional property of the polypeptides is unknown. With respect to % variants/ fragments, the only factor present in the

claim is a partial nucleic acid structure in the form of a recitation of percent identity, and there is not even structural identification of the polypeptide from which variants are derived, or identification of any particular portion of the structure that must be conserved. Accordingly, in the absence of sufficient recitation of distinguishing identifying characteristics, the specification does not provide adequate written description of the claimed genus. The Office Action also argues that the recited limitation “functions as a member of the IL-8-like chemokine family” is not meaningful because it is unclear what “IL-8-like chemokine family” encompasses, and no specific functional property for the INSP094 polypeptides of SEQ ID NO: 10 and 12 has ever been disclosed. Applicants traverse the rejection.

With respect to the argument that the recited limitation “functions as a member of the IL-8-like chemokine family” is not meaningful because it is unclear what “IL-8-like chemokine family” encompasses, and no specific functional property for the INSP094 polypeptides of SEQ ID NO: 10 and 12 has ever been disclosed, Applicants respectfully submit that this is not, in fact, the case. As set forth at page 5, lines 17-19, “functions as a member of the IL-8 like chemokine family” refers to polypeptides that comprise amino acid sequence or structural features that can be identified as conserved features within the polypeptides of the IL-8 like chemokine family. As set forth in the Examples, the claimed polypeptides are expected to fold in the same fashion as MIP-1 β . Thus, one skilled in the art would have recognized that the claimed proteins would function similarly to MIP-1 β , the member of the IL-8-like chemokine family to which the claimed protein is most related in view of the teachings of the as-filed specification.

Applicants further submit that the newly presented claims include a recitation of biological activity in association with percent identity (as compared to the full length of SEQ ID NO: 10 or SEQ ID NO: 12) and that such a recitation fulfills the requirements of the written description requirement. Accordingly, reconsideration and withdrawal of the rejection of record is respectfully requested.

Claim 46 has been rejected under 35 USC §112, second paragraph, as indefinite. In view of the cancellation of claim 46, it is respectfully submitted that the issues raised in the previous Office Action are now moot and reconsideration and withdrawal of the rejection of record is respectfully requested. With respect to the issue noted with respect to recitation of “functions as a member of the

IL-8 like chemokine family”, Applicants respectfully submit that this phrase is definite. As noted above, “functions as a member of the IL-8 like chemokine family” refers to polypeptides that comprise amino acid sequence or structural features that can be identified as conserved features within the polypeptides of the IL-8 like chemokine family. As set forth in the Examples, the claimed polypeptides are expected to fold in the same fashion as MIP-1 β . Thus, one skilled in the art would have recognized that the claimed proteins would function similarly to MIP-1 β , the member of the IL-8-like chemokine family to which was most related in view of the as-filed specification. Accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

Claim 46 has been rejected under 35 USC §102(e) as being anticipated by Ratcliffe *et al.* (U.S. Patent No. 7,238,860). The Office Action argues that Ratcliffe *et al.* disclose a polypeptide, SEQ ID NO:342, which comprises amino acids 12-19 of the present SEQ ID NO: 10 with 100% sequence identity (see computer printout of the search results). Additionally, Ratcliffe *et al.* teach fragments of the polypeptide, which is at least 5 to about 15 amino acids (column 7, lines 10-14). Therefore, the reference anticipates the claim. Applicants respectfully submit that the cited reference fails to anticipate the claims as currently presented. As indicated in the claims, the claimed fragments of SEQ ID NO: 10 or 12 are at least nine (9) consecutive amino acids. As indicated in the as-filed specification, fragments of these sequences are any number of consecutive amino acids that are the same as part, but not all, of a given sequence (see specification at page 14, lines 14-19). Thus, the claimed polypeptides can be of any given number of consecutive amino acids, provided that the total number of consecutive amino acids is less than the total number of amino acids in either SEQ ID NO: 10 or 12. Accordingly, Applicants respectfully request reconsideration and withdrawal of this rejection.

In view of the foregoing remarks and amendments to the claims, Applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 or 1.17 as required by this paper to Deposit Account 19-0065.

Applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,



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Attachment: Pisabarro *et al.*, 2006

Cutting Edge: Novel Human Dendritic Cell- and Monocyte-Attracting Chemokine-Like Protein Identified by Fold Recognition Methods

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Chemokines play an important role in the immune system by regulating cell trafficking in homeostasis and inflammation. In this study, we report the identification and characterization of a novel cytokine-like protein, DMC (dendritic cell and monocyte chemokine-like protein), which attracts dendritic cells and monocytes. The key to the identification of this putative new chemokine was the application of threading techniques to its uncharacterized sequence. Based on our studies, DMC is predicted to have an IL-8-like chemokine fold and to be structurally and functionally related to CXCL8 and CXCL14. Consistent with our predictions, DMC induces migration of monocytes and immature dendritic cells. Expression studies show that DMC is constitutively expressed in lung, suggesting a potential role for DMC in recruiting monocytes and dendritic cells from blood into lung parenchyma. The Journal of Immunology, 2006, 176: 2069–2073.

The chemokine family is a large group of small cytokines that regulate cell trafficking (1). They may share low sequence identity (identities vary from <10% to >90%) but a highly conserved three-dimensional structure (2). Chemokines contain one or two disulphide bridges characteristic of their fold and are grouped in four categories based on the arrangement of their conserved cysteines: CXC, CC, C, and CX3C.

The role of chemokines has been best investigated in the immune system. They attract Ag-capturing and APC to tissues like skin and mucosal surfaces, which are primary sites of entry for pathogens to ensure immunosurveillance (3). To elicit an immune response after an infection with a pathogen, chemokines guide APC to secondary lymphoid organs, the focal meeting points of these cells with cells of the adaptive immune response, T and B cells. Furthermore, chemokines regulate homing of lymphocyte subtypes to subcompartments of lymphoid organs,

a key function to ensure a coordinated immune response. Besides regulating cell trafficking, some chemokines have also been shown to play a role in processes such as angiogenesis and tumor growth (4, 5).

Many chemokines have been identified by their sequence signature motifs using sequence homology searches in databases (1). However, sequence homology-based methods fail as protein families become more diverse and remote homologues are difficult to identify below 20% sequence identity (6). Threading techniques utilize protein structural information to detect protein compatibility with known protein structures and, because they do not rely on sequence comparison, they are able to identify relationships even if sequence similarity is extremely low (7). In this study, we report the identification of a potential novel chemokine, DMC² (dendritic cell (DC) and monocyte chemokine-like protein), by threading methods and its functional characterization.

Materials and Methods

Sequence identification and characterization

DMC (AY358433) sequence was previously identified (8) but sequence analysis (BLAST & Pfam) failed to identify any statistically significant sequence homology with any previously characterized protein.

Generating structure-based protein function hypothesis

The fold recognition algorithm ProHit (ProCeryon Biosciences) and a fold library consisting of 7950 representative three-dimensional (3D) protein structures from the Protein Data Bank were used. Sequence-structure alignments were generated with the Smith-Waterman algorithm (9). Two mean force potentials describing the energetic forces between the residues of a fold and between residues and the surrounding solvent were used to calculate the sequence-structure fitness (10, 11). Default ProHit values for gap restrictions were used to control the number, size, and placement of gaps in the query sequence and the fold library entries. The BLOSUM40 amino acid substitution matrix was used for sequence comparison and scoring (12). For ranking and scoring we used: 1) energy score derived from residue-residue and residue-solvent interactions (pair/surf), 2) sequence similarity, 3) a normalized combination of pair/surf and sequence similarity (threading index (Th. Idx.)), and 4) ratio between the fold

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² Abbreviations used in this paper: DMC, dendritic cell and monocyte chemokine-like protein; DC, dendritic cell; 3D, three-dimensional.

length (fold length) and the number of aligned residues in the sequence-structure alignment (path length) (*fl/p*). Results were ranked by their *Th1dx.fl/p* was used to exclude sequence-structure alignments not covering the full length corresponding to a specific fold and to rule out possible false positives. Based on previous studies (data not shown), hits with values $0.6 \leq fl/p \leq 1.3$ were considered of a higher confidence. Corresponding 3D models were generated, and their respective sequence-structure alignments analyzed (ProHit and InsightII; Accelrys). The structural classification scheme SCOP (13) was used to build up a fold library containing all members of the IL-8-like fold family and to generate structure-based protein function hypothesis.

DMC protein expression and purification

His-tagged DMC was extracted from *Escherichia coli* inclusion bodies and purified on a Ni-NTA metal-chelate column. The Ni-NTA pool was applied onto a HiLoad 16/60 Superdex 75 gel filtration column (Amersham Biosciences) equilibrated with 20 mM MES (pH 6.0) containing 6 M guanidine HCl. Five milliliters of the Superdex 75 pool was treated with 50 mM DTT at pH 8.0, loaded onto a RP-HPLC Vydac C₄ column equilibrated with 0.1% trifluoroacetic acid in water, and eluted with a gradient of acetonitrile (25–37%) in 0.1% trifluoroacetic acid. DMC was lyophilized and dissolved in 1 mM HCl before dilution into assay buffer. A mutant form of DMC where all cysteines were converted to serines was generated. This His-tagged version was expressed and subjected to the same purification procedure as the wild-type protein. For baculovirus expression, His-tagged DMC was cloned into the plasmid p.HIF and transfected into SF9 cells using Lipofectin (Invitrogen Life Technologies). After a 12-h culture in Hanks' serum-free medium and 5 days in complete Hanks' medium (Invitrogen Life Technologies), SF9 cells were infected with supernatant to generate a viral stock. A second amplification was done by infecting H5 cells in ESF921 medium (Expression System LLC) and DMC was purified using a Ni-NTA column.

Circular dichroism

DMC and CCL5 (R&D Systems) were dissolved in 1 mM HCl and diluted into PBS. Circular dichroism (CD) spectra were obtained in the far-UV range (190–290 nm) using quartz cuvettes of 1-mm path length (Aviv model 62DS CD spectrometer; Aviv Associates). DMC was measured at 0.5 μ g/ml and CCL5 at 0.1 mg/ml. Data were collected at 2.0-nm intervals with bandwidth 1.0 nm and at 25°C.

Cell isolation, culture, and treatments

Human PBMC were isolated by Hypaque-Ficoll density centrifugation and cultured at 37°C in 5% CO₂ at 10⁶/ml in RPMI 1640, 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin, and streptomycin, either unstimulated or activated with 20 μ g/ml LPS (Sigma-Aldrich) for 48 h.

Ab generation and screening

Anti-DMC mAbs were generated by immunizations of mice with recombinant His-tagged DMC. For screening of the Abs, ELISA plates (Immunoplate Maxi-sorp; Nunc) was coated with 1 μ g/ml DMC or with 1 μ g/ml His-tagged control protein. After washing, the plate was incubated for 1 h with 50 μ l of the test samples, normal mouse serum, anti-DMC Ab, or serum from mice immunized with his-tagged DMC, washed again, and followed by incubation with HRP-conjugated goat anti-mouse IgG Fc (catalog no. A-168; Sigma-Aldrich) for 1 h. The plate was washed, substrate (TMB BioFX Solution, catalog no. TMB0100-01) was added, and reaction was stopped (TMB stop solution, catalog no. BSTP-0100-01; BioFX) as color developed.

Transwell migration assays and flow cytometry

PBMC transmigrated across 5- μ m Transwell migration filters (Corning) for 2.5 h in response to a stimulus in the bottom chamber and were enumerated by flow cytometry. CXCL12 (R&D Systems) was used at 0.5 μ g/ml. Subsets of migrated cells were analyzed by preblocking Fc receptors with human IgG (1 μ g/10⁶ cells; Sigma-Aldrich), followed by staining with Abs to CD3, CD14, CD11c, and CD16 (BD Biosciences) and analysis on a FACScan. Cells were preincubated for 30 min at 37°C in 5% CO₂ with pertussis toxin (List Biological Laboratories). Mouse mAbs against DMC were generated in-house and used at 10 μ g/ml. Anti-CCL2 Ab (clone 24822; R&D Systems,) was used at 10 μ g/ml.

Northern blot analysis

DMC expression was analyzed with commercially available multiple human tissue Northern blots (BD Clontech).

Immunohistochemistry

Formalin-fixed, paraffin-embedded adult normal and inflamed lung, normal colon, and small intestine tissues were used for immunohistochemistry. Tissue

sections were deparaffinized and hydrated. For Ag retrieval, slides were incubated in two rounds of Trilogy Ag retrieval solution (Cell Marque) at 99°C for 30 min. Endogenous peroxidase activity was quenched with 3% H₂O₂, then blocked with Vector Biotin Avidin Blocking reagents (Vector Laboratories), and the slides were blocked with 10% horse serum. Sections were stained with an in-house generated mouse anti-DMC mAb (clone 3H8) at 10 μ g/ml, followed by a biotinylated horse anti-mouse Ab (Vector Laboratories) diluted 1/200 in blocking serum. For detection, Vector's ABC kit was used and metal enhanced diaminobenzidine (Pierce). Sections were counterstained with Mayer's hematoxylin.

Results and Discussion

Assigning remote homologies

DMC is a novel secreted protein of unknown function (8). It is a basic protein (pI \approx 10.9) of 119 aa and an estimated molecular mass of 13,819 Da. The putative signal peptidase I cleavage site is between positions 23 and 24 (Fig. 1A) and it has no potential Asn-linked glycosylation sites. Its chromosomal location is 19:47,624,876–47,638,824.

Sequence similarity searches only identify a murine ortholog (71% identity; Fig. 1A). To search for structural homologies,

A

Human DMC	1	M K V T I S R L E L L L P L M D M S M V S S L N P	26
Mouse DMC	1	M K L A S P F L L L P V H L N S V S S S P H P	26
CXCL8	1	M T S R D A V A I L A F L I S A A L C H G A	31
CXCL14	1	M S L P R R A P P V S H L L A A A L L L L L	25
Human DMC	27	V Y A R G R R D R G Q A R R R W L Q S S G Q E C E C	52
Mouse DMC	27	G V A R S H G D Q H L A P R W L L R G G Q E C E C	52
CXCL8	24	V L P R S A K E L R C Q C	36
CXCL14	26	A L Y T A R V D R K C C	39
Human DMC	53	K D F L R A P R R K E M T V S S L P R K . . Q P	76
Mouse DMC	53	K D P L Q A P K R K A T A V L G S P R K . . Q C P	76
CXCL8	37	I K T V S R P P P P P F K E L K E L R V I S S G P H C A	62
CXCL14	40	S R K G P K Y D V A D V K R L E M K P R Y P . H C	64
Human DMC	77	D H E K G R V K K T A L Q R H R K K P N	97
Mouse DMC	77	D H V E G R E K K N R E K H H P R E Q Q	97
CXCL8	63	H T E I I V H L S D G S E L L O P K E N	81
CXCL14	65	E K M V I T T K S V S R Y R G Q E H C L R P K L Q	96
Human DMC	98	K H N R A C C Q L R K Q C G L R S P A L P L	119
Mouse DMC	98	R P S R A C Q Y L K R H L A S A A P L	119
CXCL8	84	S V Q R V E L F L E R A E	97
CXCL14	91	S T H K Y I H W Y H A N S E M A R V S E . .	111

B

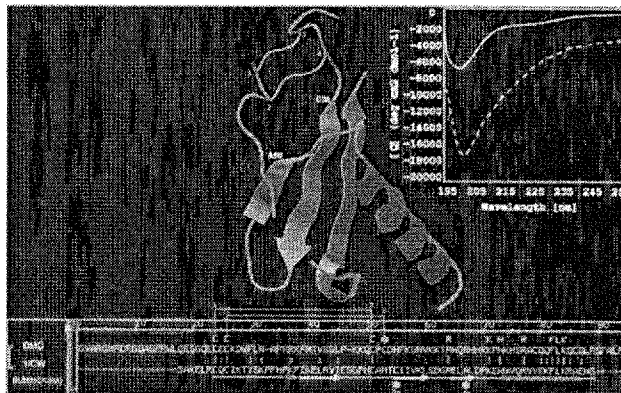


FIGURE 1. A, Sequence alignment of human DMC, mouse DMC, and human CXCL8 and CXCL14. Cysteine residues forming disulfide bonds are displayed in boxes. Other cysteine residues are underlined. Signal sequences (as predicted by SignalP 3.0) are shown in italics. B, 3D model of DMC and sequence-structure alignment with 1ICW (IL8_{E38C/C50A}). Green ribbons are α helices and yellow arrows β strands. Cysteine residues are highlighted and disulfide bridges (C1–C3; C2–C4) are marked as red lines. In IL8_{E38C/C50A}, C4 corresponds to residue 38 and in IL-18 to residue 50 (white stars in alignment and labeled in 3D model). The far UV CD spectra of DMC (solid blue line) and RANTES (dashed) are shown from 195 to 260 nm in the upper right panel.

DMC's sequence was threaded against a fold library of 7950 3D protein structures, obtaining a structural model for each fold. From the 20 top hits (Table 1; see *Materials and Methods* for scoring and ranking), 9 were considered of "high confidence level," of which only hits 6 and 12 were taken as "true positives" for not having high content of gaps, not lacking any secondary structure motif in the sequence-to-structure alignment, and presenting good matching of cysteines. Hits 6 and 12 correspond to IL-8-like chemokines (CCL3 and CXCL8, respectively). Based on these structural similarities, we assigned an IL-8-like fold to the DMC sequence. Secondary structure predictions performed for DMC (PHD, DSC; data not shown) are in agreement with an IL-8-like fold. Src homology 2 (SH2)-like hits were considered false positives based on the fact that they had secondary structure elements missing in their alignments to DMC.

To assess accuracy of our structural hypothesis, we constructed a fold library containing all of the known IL-8-like structures (SCOP; data not shown). DMC and, as control, sequences of known IL-8-like proteins were threaded against this fold library, showing threading scores in the same range (sequence identity 8–16%; data not shown). The highest scores obtained for DMC were with a CXCL8 mutant (IL8_{E38C/C50A}; 1ICW PDB), which lacks the characteristic cysteine pattern of IL-8-like proteins but still adopts an IL-8-like fold and has functional chemokine properties (14). DMC contains six cysteines. Graphical analysis of the potential disulphide bridges of DMC on the generated 3D 1ICW-like model allowed us to observe that the first three cysteines of DMC align with those of CXCL8 and 1ICW, and that the fourth cysteine in DMC is localized in a different position in sequence but equivalent in three dimensions to a cysteine in 1ICW (Fig. 1). We concluded that DMC disulfide bridges may be structurally similar to those in 1ICW and CXCL8 and that they may help DMC to fold as a CXCL8-like protein, suggesting that DMC is a novel CXC chemokine-like protein. Structural similarities between some members of the IL-8 chemokine family were experimentally as-

sessed by CD. Active, purified DMC protein adopts a secondary structure (Fig. 1B, *upper right panel*) similar to that observed for CCL5 and CCL4, both members of the IL-8-like fold family (15).

These findings extend the CXC chemokine signature and may help to identify novel members of the group.

DMC specifically induces migration of monocytes and DCs

To test whether human DMC shows chemotactic activity, we expressed his-tagged DMC in *E. coli* and performed Transwell migration assays with human PBMC. DMC specifically induced migration of CD14⁺ monocytes and CD14⁺CD11c⁺ DCs (Fig. 2A). Other PBMC subtypes, such as CD3⁺ T cells, CD16⁺CD3⁺ D14⁺ neutrophils, and NK cells (Fig. 2A), or B cells (data not shown) were not attracted by DMC. As seen for other chemokines, the dose-response curve of monocyte and DC migration to DMC in the migration assay formed a bell-shaped response curve. Because proteins expressed in *E. coli* are not always folded correctly, we also expressed his-tagged DMC protein in the baculovirus system and confirmed the chemoattraction of monocytes and immature DCs (Fig. 2B). A similar migration activity was observed with a nontagged version of DMC (data not shown). To investigate whether DMC also recruits activated DCs and monocytes, PBMC were stimulated with LPS in cell culture before migration assays. LPS completely inhibited migration of DCs (Fig. 2C) and monocytes (data not shown) to DMC. Furthermore, activation of monocytes with PGE₂ and forskolin significantly reduced the response to DMC (data not shown). Heat inactivation of DMC abolished migration of nonactivated DCs and monocytes (data not shown), as did mutation of all cysteines of DMC into serines (Fig. 2D).

The receptor to DMC is currently unknown, and there is no evidence that any of the known CXC receptors might also function as a receptor for DMC as tested in migration assays by addition of Abs to the various CXC receptors (data not shown). However, migration of DCs and monocytes was inhibited by

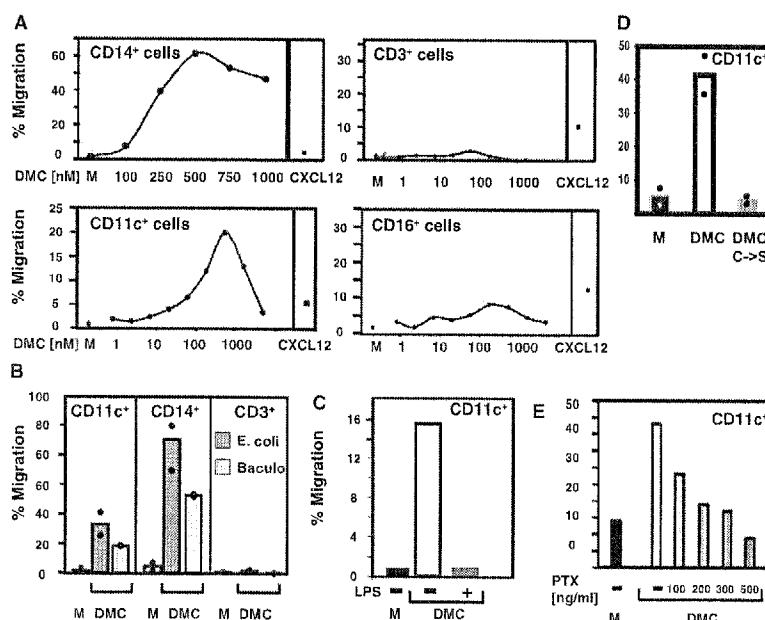
Table 1. Threading results and summary of structural and functional hypothesis

	Thr. Id.	% Id. ^a	pl	fl	fl/pl	cl ^b	Protein Data Bank Hits	Structural/Functional Classification
1	36.34	25.0	72	84	1.17	HC	1dsz.B	RXR- α DNA-binding domain
2	31.33	24.6	65	282	4.34	FP	1aj2	Dihydropterolate synthase (TIM β/α barrel)
3	30.20	17.7	79	104	1.32	FP	1d4t.A	T cell signal transduction molecule SAP SH2 domain
4	30.18	18.2	88	363	4.12	FP	1ga0.A	β -Lactamase/transpeptidase-like
5	29.15	18.5	92	130	1.41	FP	1jsf	Lysozyme
6	28.64	16.2	68	70	1.03	HC	1ha6.A	MIP (IL-8 like)
7	27.78	20.0	90	324	3.60	FP	1hqt.A	Aldehyde reductase
8	27.49	26.5	49	66	1.35	FP	5gat.A	Erythroid transcription factor (DNA-binding domain)
9	26.49	16.1	81	103	1.27	HC	1sha.A	v-SRC tyrosine kinase SH2 domain
10	25.84	25.0	36	40	1.11	HC	1fmy.A	Metallothionein
11	25.16	23.8	63	143	2.27	FP	1ehw.A	Nucleoside diphosphate kinase (Ferredoxin like)
12	24.89	14.3	70	68	0.97	HC	3il8	IL-8
13	24.89	18.6	86	479	5.57	FP	1ew2.A	Phosphatase
14	24.72	18.5	81	106	1.31	FP	1aou.F	SH2 domain FYN tyrosine kinase
15	24.57	19.3	57	61	1.07	HC	1nea	α toxin
16	24.52	13.6	88	107	1.21	HC	3hck	SH2 domain hemopoietic cell kinase HCK
17	24.40	19.6	51	51	1.00	HC	1ha8.A	Pheromone ER-23
18	24.19	16.1	81	450	5.55	FP	2sre	c-SRC tyrosine kinase SH2 domain
19	24.01	13.1	84	109	1.30	HC	5pal	Parvalbumin (EF hand like)
20	23.64	19.8	86	130	1.51	FP	1ivm.A	Lysozyme M

^a Percent sequence identity.

^b cl, Confidence level HC, high; FP, false positive.

FIGURE 2. Transwell migration assays of PBMC in response to DMC. Results are presented as percentage of input cells of each cell subtype migrating to the lower chamber of a Transwell in response to the indicated concentrations of DMC. M, Medium alone. *A*, Migration of PBMC subsets: CD14⁺ monocytes, CD3⁺ T cells, CD11c⁺CD14⁺ DCs, CD16⁺ cells containing granulocytes and NK cells. *B*, Migration of CD11c⁺ DCs, CD14⁺ monocytes, and CD3⁺ T cells in response to DMC (0.5 μ M) expressed in *E. coli* or in baculovirus. *C*, Migration of CD11c⁺ DCs in response to DMC (0.5 μ M) after preincubation for 48 h with medium alone or LPS. *D*, Mutation of all cysteines into serines (C \rightarrow S) abolishes DMC activity in migration assays. *E*, Inhibition of DMC-induced migration of DCs and monocytes by pretreatment of PBMC with pertussis toxin.



PTX (Fig. 2*E*), indicating that the receptor for DMC is a seven-transmembrane G α i protein-coupled receptor.

A panel of monoclonal mouse anti-DMC Abs was generated and selected by binding to DMC but not to the his tag (Fig. 3*A*). A subset of the Abs was further characterized, and several Abs were found to completely inhibit migration of monocytes and DCs to DMC as shown for clone 3H8 (Fig. 3*B*). Clone 3H8 is an IgG1 Ab with a κ L chain. It also recognized DMC specifically by Western blot (data not shown). Anti-CCL2 Abs did not block migration to DMC (Fig. 3*B*). Furthermore, DMC protein bound specifically to monocytes and DCs but not to T cells as detected by flow cytometry using the monoclonal anti-DMC Ab and freshly isolated PBMC (data not shown).

The majority of other monocyte-attracting chemokines preferentially induce migration of activated monocytes (16). CCL2 is specific for attraction of activated monocytes to sites of inflammation (17), and CXCL14 preferentially attracts monocytes activated with PGE₂ or forskolin (18). CX3CL1, which is

expressed by bronchiolar epithelial cells in chronic granulomatous inflammation of the lung, attracts subsets of monocytes (19).

Circulating blood DCs express a variety of chemokine receptors, CCR1, CCR2, CCR3, CCR5, and CXCR4 (20). They have been shown to respond to the CC chemokines CCL2, CCL8, CCL13, CCL5, and CCL11, which allows their recruitment to sites of inflammation (20). Migration of blood DCs to noninflammatory sites may be regulated by CXCL12, an ubiquitously expressed chemokine (21). CCL20, a chemokine expressed in noninflamed lung and liver, may recruit certain subsets of immature DCs into these tissues (22). However, unlike immature DCs generated *in vitro* from CD34⁺ bone marrow cells, blood DCs do not express CCR6 and therefore do not respond to CCL20 (23). We propose that DMC may fulfill this role.

DMC expression

Northern blot analysis using human multitissue blots showed that DMC is expressed in adult trachea, stomach (Fig. 4*A*), and fetal lung (Fig. 4*B*). Immunohistochemistry analysis of adult normal lung tissue sections demonstrated that DMC is constitutively expressed on bronchial and bronchiolar epithelium (Fig. 4*C*), as well as in a subset of alveolar lining cells (Fig. 4*E*). In addition, DMC expression was also observed in lung tissue from patients with asthma or obstructive pulmonary disease (data not shown). The expression levels and pattern were similar regardless of the inflammation status of the lung, suggesting that chronic inflammatory processes do not regulate DMC expression. However, acute inflammatory conditions have not been investigated yet. Furthermore, DMC expression was also observed in adult, normal small intestine (duodenum) and colon tissue sections (Fig. 4, *G* and *I*). Specifically, DMC staining is detected in the villus and some crypt epithelial cells of the small intestine (Fig. 4*G*) and in colonic epithelial cells, primarily at the luminal side (Fig. 4*I*). Constitutive expression of DMC in lung, stomach, colon, and small intestine supports a

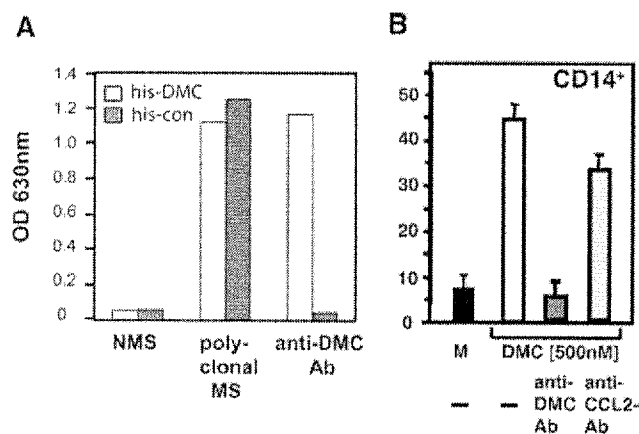


FIGURE 3. *A*, ELISA testing specific binding of generated mouse anti-human DMC Abs (clone 3H8) to DMC, but not to the his-tag. *B*, mAbs (3H8) against DMC inhibit migration of monocytes and DCs to DMC. Anti-CCL2 is used as a control Ab.

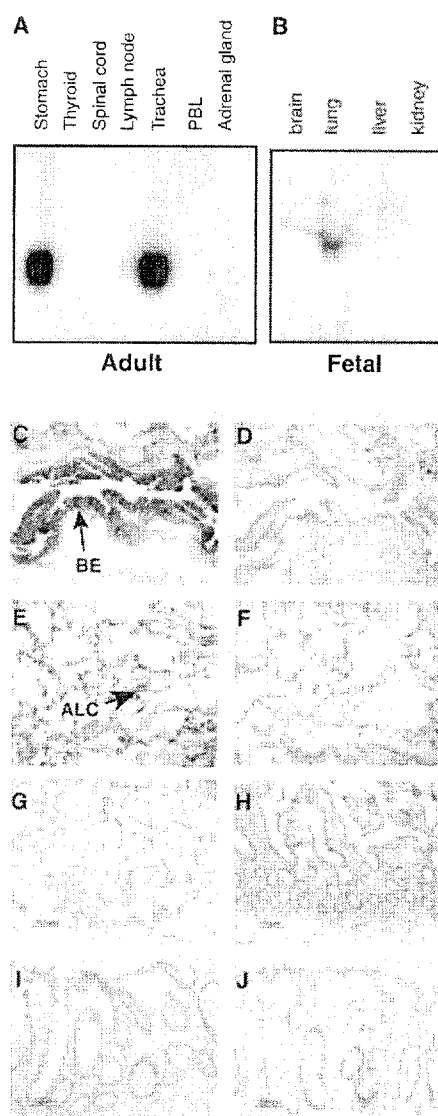


FIGURE 4. Expression pattern of DMC in normal human tissues. Northern blot analysis of total RNA from adult (A) and fetal (B) human tissues probed to detect DMC. C–F, Expression of DMC in adult human lung. IHC analysis of normal lung sections stained with an anti-DMC Ab (C and E) or an isotype control Ab (D and F). DMC is detected in human bronchiolar epithelium (BE) and a subset of alveolar lining cells (ALC). Stainings are representative of 26 lung samples from patients with asthma, chronic obstructive pulmonary disease, or normal lungs. G–J, Expression of DMC in adult human small intestine (duodenum) and colon. Sections were stained with an anti-DMC Ab (G and I) or an isotype control Ab (H and J). DMC is detected in small intestine villus and some crypt epithelial cells (G). DMC is also detected in colonic epithelial cells (I), primarily at the luminal surface. Stainings are representative of three normal patients for each tissue type.

potential role for DMC as a housekeeping chemokine regulating recruitment of nonactivated blood monocytes and immature DCs into tissues. The presence of APCs at mucosal surfaces of these tissues is of biological importance for immunosurveillance of potentially harmful pathogens that may enter the lungs with air intake and the intestinal tract via food intake. If a pathogen invades, it will quickly activate the local APCs. Con-

sequently, the local DCs mature, stop responding to DMC, and migrate to secondary lymphoid organs, where they activate the adaptive immune response to eradicate the pathogen.

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Disclosures

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